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13. ABSTRACT (Maximum 200 words) This project is studying the development of dendritic cell vaccines for immune therapy of breast cancer. We have developed techniques for isolation and immunogen loading of murine splenic dendritic cells which induced humoral and cellular immunity in a model system (BSA). Translation to a tumor antigen (CEA) produced weak immune responses and antitumor effects. This system requires large amounts of immunogen to be effective. An alternate approach of developing gene transfer techniques to induce immunogen expression in dendritic cells is ongoing. We have developed a vector system using replication defective adenoviruses which is able to transfect dendritic cells as demonstrated with luciferase (reporter gene). We have constructed a CEA vector (Ad-CEA) which induces CEA expression in cells (<i>in vitro</i>); it induced antibody to CEA following IV and IP injection but not antitumor effects (TH-2 response). Generation of Ad-CEA dendritic cell vaccines are ongoing and already show evidence of CEA immune responses. We will examine these Ad-CEA dendritic cell vaccines as therapy in metastatic breast cancer models and examine strategies to switch the Ad-CEA (alone) system to a more effective immune response (TH-1 response). We are constructing adenovirus-Erb-2 constructs as a second breast cancer relevant system.				
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Albert F. R. Anglin 9-5-95
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Introduction:

The underlying reason for failure to cure patients diagnosed with breast cancer is the presence of micro-metastases in approximately one-third of breast cancer patients (1,2). The stimulation of antitumor immune responses represents one of the most effective ways to treat low tumor burdens that are microscopically or clinically occult (3,4). The objective of our proposal is to determine whether novel dendritic cell (DC) vaccines containing a human tumor-associated antigen (carcinoembryonic antigen) can induce systemic immunity and eradication of micro-metastases in a syngeneic murine breast cancer model which expresses human CEA. The feasibility and efficacy of this CEA vaccine strategy would be applicable to other putative tumor-specific or associated tumor antigens.

We propose to develop dendritic cell vaccines composed of syngeneic dendritic cells which have been manipulated *ex vivo* to contain carcinoembryonic antigen (CEA). Several methods will be used to place pure CEA in the cytoplasm of these cells, i.e., passive endocytosis, acid labile liposomes and gene transfer. It is our hypothesis that CEA containing dendritic cells will be a potent induction mechanism for cellular and humoral immunity to CEA. We will characterize their ability to induce CEA specific immune responses *in vivo*. Using a syngeneic murine breast cancer model which expresses human CEA, we will characterize the ability of these vaccine reagents to protect against tumor challenge and to treat animals harboring occult micro-metastases.

Body:

The first year of this proposal has consisted of the examination and manipulation of dendritic cells for the purpose of developing a potent, novel tumor vaccine that will be applicable to human clinical trials. Dendritic cells are highly potent antigen-presenting cells (APCs) able to initiate and stimulate cellular immune response *in vitro* and *in vivo* (5). Initiation of immune response to an antigen clearly includes the role of APCs responsible for intracellular antigen processing with subsequent cell-contact mediated stimulation of naive T cells (6). Of the several types of APCs (macrophages, monocytes, activated B cells, etc.), dendritic cells have been shown to be the most potent or effective with estimates of 40 to 100-fold increments in activity over macrophages or B cells (7,8). Dendritic cells appear operative in initiation of immune responses to transplantation antigens, soluble proteins, viral infections, etc. (9). They are capable of activity in both primary and secondary immune responses with a large body of *in vitro* studies and increasing observations *in vivo* (8,10). In this regard, tumor vaccine studies employing autologous tumor cells transduced with genes encoding a variety of cytokines demonstrated the greatest immunoprotection and therapy using GM-CSF (a dendritic cell growth factor).

Furthermore, techniques currently exist for isolation and *in vitro* culture of human dendritic cells so that strategies of dendritic cell vaccines can be readily translated to human trials. We have become adept at isolating dendritic cells from mouse spleens using the procedure of Steinman et al. (10,11). Briefly, spleens are collagenase-digested and passed through a cell strainer. The dendritic cells are then floated over a dense bovine albumin gradient and selected by nonadherence to plastic tissue culture plates after overnight culture. To examine loading of

dendritic cells with soluble protein antigens as a means of immunization, we needed to devise a dendritic cell enrichment procedure which avoids exposure to heterologous proteins such as bovine albumin and fetal calf serum. We successfully modified the enrichment procedure of Steinman et al. (10,11) to eliminate protein exposure except of the specific protein being studied by floating dendritic cells over a Percoll gradient (instead of BSA) and use of rat serum in the overnight culture instead of fetal calf serum.

Dendritic cells are equipped with cell surface molecules which enable their role in triggering T helper and cytolytic T cell responses. Highly purified dendritic cells have a rich display of both class I and II major histocompatibility complex (MHC) molecules (6,12) as well as a potent array of co-stimulatory molecules including LFA-3, ICAM-1 and B7/BB1 (13). These cell surface molecules stimulate naive mononuclear cells from other murine strains to proliferate in the mixed lymphocyte reaction. We have verified the quality of our dendritic cell preparations using this assay and have found them to be 100-fold more potent than whole splenic mononuclear cells.

Dendritic cells exposed to soluble protein antigens can mediate T cell immune responses both *in vitro* and *in vivo*. Dendritic cells (prior to prolonged culture) can take up soluble proteins, process them and induce T cell responses *in vitro* reflecting immune T cell receptor interaction with class II MHC-peptide complexes (7). *In vivo* studies have also shown that such *in vitro* "pulsed" dendritic cells can induce immune T cell and antibody responses (10,11).

In seeking to exploit these properties of dendritic cells to develop a vaccination strategy, we initially examined the ability of protein-pulsed dendritic cells administered by various routes and at varying doses to elicit humoral and cellular immune responses. We decided to do these initial studies using immune response to bovine serum albumin (BSA) given the need for large amounts of pure protein for such studies.

**Table 1. Anti-BSA Antibody Response Elicited by BSA-Pulsed Dendritic Cells
Delivered by Various Routes^a**

Route	Group 1		Group 2	
	Day 28	Day 35	Day 56	Day 63
Intraperitoneal	450 ± 200 ^b	13,000 ± 6,000	3,300 ± 1,000	110,000 ± 50,000
Intravenous	1,200 ± 200	160,000 ± 40,000	22,000 ± 7,000	260,000 ± 10,000
Intradermal	1,100 ± 100	23,000 ± 8,000	4,400 ± 1,000	99,000 ± 30,000

^a Groups of five mice received 500,000 BSA-pulsed DCs by various routes on day 1 followed by BSA protein boost on day 28 (Group 1) or DCs days 1 and 28 with BSA protein boost on day 56 (Group 2).

^b Values are ng ¹²⁵I-BSA bound/ml of sera ± S.E.M. A positive result is defined as exceeding the mean +2 S.D. of a panel of normal control sera and is >60 ng/ml.

At a dose of 500,000 BSA-pulsed DCs, the antibody titers of three routes, intraperitoneal (i.p.), intravenous (i.v.) and intradermal (i.d.), were clearly positive and were comparable to each other four weeks after the initial cellular injection (Table 1). All three groups had titers which were much greater than the control mice who received MNCs exposed to BSA and washed three times (data not shown). Given a BSA challenge of 100 μ g subcutaneously, the mice who received cells via the i.v. route had a log-fold greater antibody response to BSA than either the i.p. or i.d. routes seven days post-challenge. This is despite the fact that the i.v. mice received no incomplete Freund's adjuvant (IFA) with the BSA challenge, while the i.p. and i.d. mice did receive IFA with the BSA. This is significant because naive mice show a greater antibody response at seven days to BSA in IFA than to straight BSA protein (700 ± 90 and 220 ± 60 , respectively).

After receiving the initial cellular injection followed by a boost injection of cells four weeks later, the titers of all three groups were significantly higher than the groups who received only one injection of cells. The mice who received cells i.v. responded to the protein challenge the best, with antibody titers 6-fold greater than either the i.p. or i.d. mice. Seven days after being challenged with BSA, the antibody titers of the i.p. and i.d. groups approached that of the i.v. group. They were all the same order of magnitude, yet the i.v. group had a significantly higher titer, again without IFA in the challenge.

We tested different doses of DCs with the i.p. route (Table 2).

Table 2. Anti-BSA Antibody Response Elicited by Various Numbers of BSA-Pulsed Dendritic Cells^a

Cell Type and Dose	Group 1				Group 2			
	Day 28		Day 35		Day 56		Day 63	
500,000 MNCs	2 \pm	1 ^b	1,100 \pm	300	210 \pm	50	2,400 \pm	1,000
500,000 DCs	450 \pm	200	13,000 \pm	6,000	3,300 \pm	1,100	110,000 \pm	50,000
250,000 DCs	510 \pm	90	1,100 \pm	900	27,000 \pm	10,000	61,000 \pm	8,000
125,000 DCs	40 \pm	20	1,600 \pm	70	110 \pm	20	17,000 \pm	5,000

^a Groups of five mice received BSA-pulsed cells by i.p. routes on day 1 followed by BSA protein boost on day 28 (Group 1) or DCs days 1 and 28 with BSA protein boost on day 56 (Group 2).

^b Values are ng ¹²⁵I-BSA bound/ml of sera \pm S.E.M. A positive result is defined as exceeding the mean +2 S.D. of a panel of normal control sera and is >60 ng/ml.

Given one injection of cells, the mice who received 250,000 or 500,000 DCs had positive antibody titers to BSA after 28 days, as seen in Table 2. The mice who received only 125,000 DCs and the control mice had negative titers after 28 days. When challenged with protein, every group of mice converted to a positive titer after seven days. The mice who received only

250,000 or 125,000 DCs had no advantage in antibody production over those who received MNCs. However, the mice who received 500,000 DCs produced antibodies at levels a log higher than the other mice. In unboosted mice, the dose of 125,000 DCs yielded lower antibody titers than the higher numbers of DCs, indicating a dose-dependent effect (day 28 values).

Twenty-eight days after the mice were boosted with the varying doses of DCs (Group 2), each group of mice had a positive titer to BSA. The group who received only 125,000 DCs had a titer actually lower than the control group who received only MNCs. The group who received 500,000 DCs had a log-fold greater titer than controls, and the group who received 250,000 DCs had a titer a log-fold greater than the 500,000 DC group. Seven days after protein challenge, all groups were definitely positive and significantly greater than the control group. At this timepoint, a dose-dependent effect is seen, with the best response seen with the group who received 500,000 cells.

Lymphoblastic transformation (LBT) assays were performed to document systemic T cell immunity to BSA following DC immunization. Specific lymphoblastic transformation to BSA was not elicited in any of these groups of mice; however, the splenic T cells did transform when re-exposed to fetal calf serum (FCS) at 1% and 10% as shown in Table 3.

Table 3. Lymphoblastic Transformation Response Elicited by BSA-Pulsed Dendritic Cells Delivered by Various Routes

Antigen ($\mu\text{g/ml}$)	Cell Type and Route							
	MNCs i.p.		DCs i.p.		DCs i.v.		DCs i.d.	
Control ^a	130 \pm	40 ^b	54 \pm	30	26 \pm	5	33 \pm	5
BSA (100)	140 \pm	100	38 \pm	20	40 \pm	10	33 \pm	6
BSA (30)	120 \pm	40	37 \pm	20	34 \pm	6	38 \pm	9
BSA (10)	130 \pm	20	34 \pm	20	50 \pm	10	59 \pm	10
FCS 10%	680 \pm	600	10,000 \pm	5,000	3,300 \pm	1,000	690 \pm	300
FCS 1%	220 \pm	100	5,100 \pm	3,000	580 \pm	400	110 \pm	30
FCS 0.1%					120 \pm	30	45 \pm	10
OVA ^c (100)	110 \pm	40	33 \pm	7	20 \pm	3	24 \pm	3
Con A	74,000 \pm	20,000	2,000 \pm	500	5,400 \pm	400	1,000 \pm	200

^a Wells received 10^5 nylon wool enriched T cells and 5×10^5 syngeneic irradiated splenocytes as antigen-presenting cells.

^b Values are cpm of ^3H -thymidine incorporated into cells \pm S.E.M.

^c OVA, ovalbumin.

The LBT assays of the three routes of administration of DCs in boosted groups at the maximum dose of 500,000 cells were compared four weeks after the last injection of cells. The group who received the cells i.p. had the greatest response to FCS, being two log-fold greater than the control group who received 500,000 MNCs. The group who received the cells i.v. had counts one log-fold greater than the controls, and the i.d. group had counts no greater than the control

group. The counts of all groups decrease in parallel with the amount of FCS in the well. All groups responded well to con A and did not respond at all to ovalbumin.

The lymphoblastic transformation assay was also used to compare the efficacy of varying doses of dendritic cells to elicit systemic T cell immunity to BSA in boosted groups who received cells i.p. Again, there was a good response to re-exposure to FCS without much response to BSA alone. A dose-dependent effect is seen in Table 4.

Table 4. Lymphoblastic Transformation Elicited by Various Numbers of BSA-Pulsed Dendritic Cells

Antigen ($\mu\text{g/ml}$)	Cell Type and Number ^a							
	500,000 MNCs		500,000 DCs		250,000 DCs		125,000 DCs	
Control ^b	130 \pm	40 ^c	54 \pm	30	33 \pm	6	35 \pm	6
BSA (100)	140 \pm	100	38 \pm	20	30 \pm	6	32 \pm	10
BSA (30)	120 \pm	40	37 \pm	20	27 \pm	5	37 \pm	7
BSA (10)	130 \pm	20	34 \pm	20	50 \pm	10	47 \pm	10
FCS 10%	680 \pm	600	10,000 \pm	5,000	2,700 \pm	1,000	140 \pm	60
FCS 1%	220 \pm	100	5,100 \pm	3,000	500 \pm	200	55 \pm	20
FCS 0.1%					90 \pm	60	33 \pm	5
OVA ^d (100)	110 \pm	40	33 \pm	7	16 \pm	7	20 \pm	3
Con A	74,000 \pm	20,000	2,000 \pm	500	4,800 \pm	800	860 \pm	200

^a All cells were injected intraperitoneally.

^b Wells received 10^5 nylon wool enriched T cells and 5×10^5 syngeneic irradiated splenocytes as antigen-presenting cells.

^c Values are cpm of ^3H -thymidine incorporated into cells \pm S.E.M.

^d OVA, ovalbumin.

An increasing response to FCS is obtained as the cell numbers increase. In the group that received only 125,000 DCs, counts are below that of background. The mice who received 250,000 DCs responded to FCS a log-fold greater than controls. All groups responded well to con A and were negative to ovalbumin.

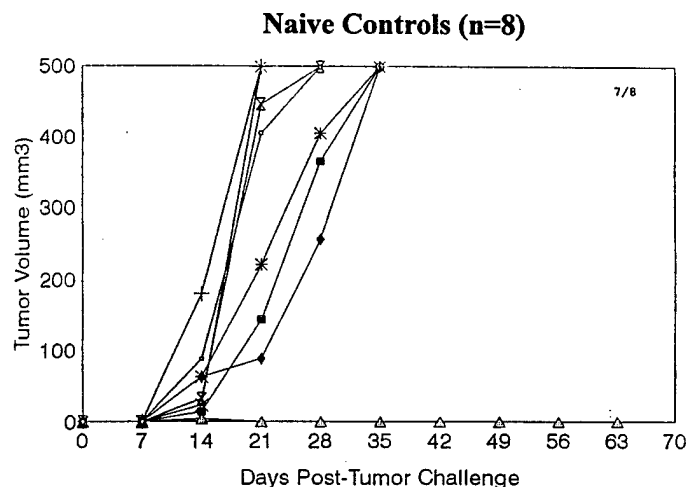
These studies utilized dendritic cells exposed to a foreign protein (BSA) in overnight culture and the injection of protein loaded cells for immunization. While many routes and doses have been examined in the literature, this is the first study to my knowledge where various routes were compared in one study. We have presented this data at the Keystone Symposium for Dendritic Cell Research (Taos, New Mexico, March 1995). Data which we have obtained regarding the optimal cellular dose and delivery route for protein-pulsed dendritic cell vaccines is applicable to dendritic cell immunization against a wide array of target antigens, including tumor-associated antigens.

Carcinoembryonic antigen (CEA) represents a reasonable tumor-associated antigen for a tumor vaccine against multiple human adenocarcinomas (breast, colon and non-small cell lung cancer)

(14,15). The CEA gene has been cloned and inserted into a vaccinia virus genome (16,17). This recombinant vaccinia-CEA (rV-CEA) vaccine induces CEA-specific humoral and cellular immune responses in mice (17).

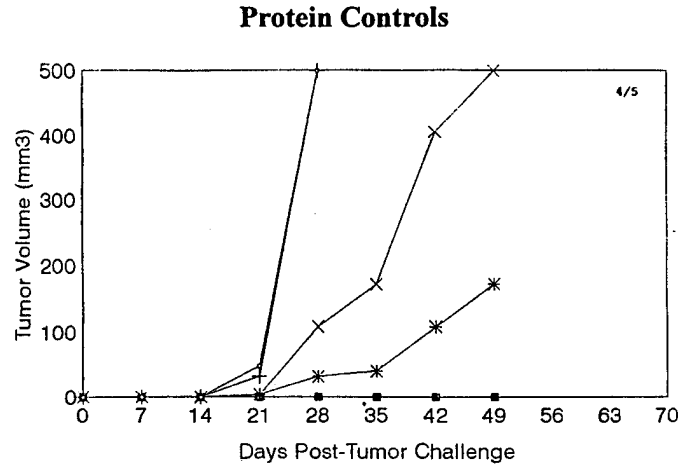
We have evaluated two strategies to develop a CEA-dendritic cell vaccine. First, we capitalized on the ability of the dendritic cells to take up, process and present antigen by exposing the cells to purified CEA protein in overnight culture. We have shown that CEA-pulsed dendritic cells can stimulate proliferation of CEA-immune T cells *in vitro*, which indicates intracellular processing and presentation of the antigen on the surface of the dendritic cells. The limited availability of purified tumor antigens including CEA requires protein loading overnight with concentrations of immunogen that are one to two logs lower than prior published studies or our BSA model. However, *in vitro* stimulation of CEA immune T cells by CEA pulsed dendritic cells suggested that *in vivo* studies were at least feasible. We were unable to induce reproducible antibody or cellular immunity to CEA in mice receiving CEA pulsed dendritic cells in various doses by i.v. and i.p. routes. Evidence for "priming" of the immune response can be seen in the tumor challenge studies provided in Figure I. Seven of eight mice challenged with 2×10^5 syngeneic CEA expressing tumor cells developed tumors (Figure I), four of five mice given a single dose of CEA protein similarly developed tumors (Figure II) while zero of eight animals developed tumors who received 500,000 CEA pulsed dendritic cells times two followed by a single dose of CEA protein (Figure III). The requirement for large doses of immunogen and the weak immune response to CEA protein pulsed dendritic cells suggested we needed an alternate strategy to generate intracellular immunogen. Analysis of liposome systems documented their need for high concentrations of immunogen as well. We thus turned to CEA gene transduction of dendritic cells.

Figure I. CEA-Pulsed Dendritic Cell Study



Control (unimmunized) mice received a subcutaneous injection of 2×10^5 MC38, CEA expressing tumor cells and had serial tumor measurements made over 63 days. Seven out of eight mice developed tumors.

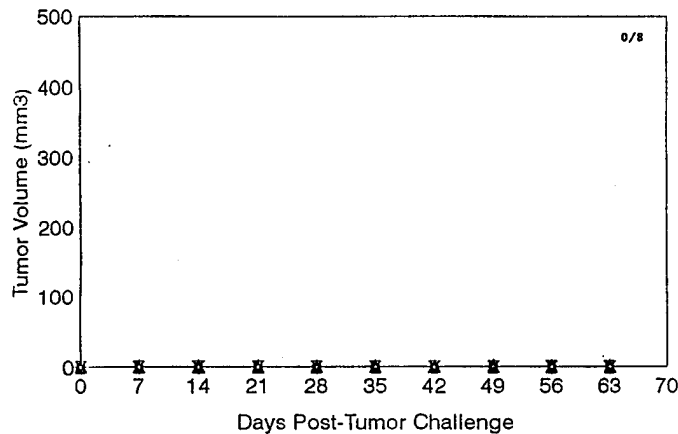
Figure II. CEA-Pulsed Dendritic Cell Study



Five mice were immunized with a single dose of 100 µg of CEA protein subcutaneously and injected subcutaneously with 2×10^5 MC38, CEA expressing tumor cells 28 days post-CEA injection and had serial tumor measurements made over 63 days. Four out of five mice developed tumors.

Figure III. CEA-Pulsed Dendritic Cell Study

500,000 DCs IP x 2 injections; CEA protein SQ at 8 weeks



Eight mice received intraperitoneal injections of 500,000 CEA pulsed dendritic cells on days 1 and 29. They then received a CEA protein boost (100 µg) subcutaneously on day 57 and were challenged with 2×10^5 MC38, CEA expressing tumor cells subcutaneously on day 85. Serial tumor measurements were made over 63 days. Zero out of eight mice developed tumors.

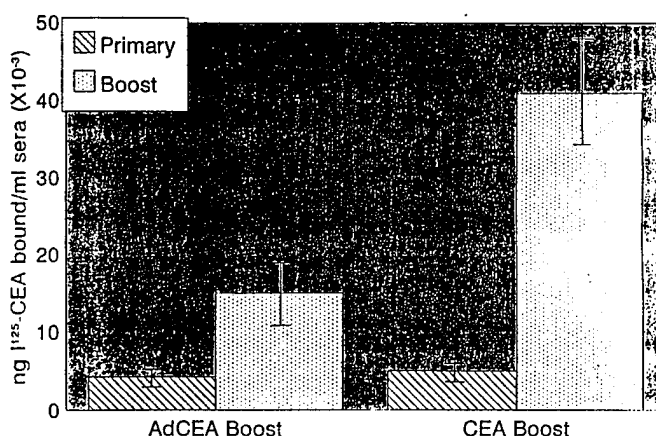
We evaluated the relative transducibility of human hematopoietic cells (T cells, B cells, monocytes, and dendritic cells) by a panel of vectors encoding a luciferase reporter gene (recombinant adenovirus, DNA/liposome complexes, RNA/liposome complexes, and DNA/adenovirus conjugates). Recombinant adenovirus was the only vector demonstrating the ability to transduce unstimulated human hematopoietic cells; and dendritic cells were the most transducible subpopulation with luciferase activity 8 to 40-fold that of T cells, B cells or monocytes (Table 5). Similar results were observed with recombinant adenovirus using murine dendritic cells. Each dendritic cell purification was verified by stimulatory activity in the mixed lymphocyte reaction (30 to 100-fold more potent than MNCs). A recombinant adenovirus encoding human CEA (Ad-CEA) was constructed and verified by transduction of carcinoma cells with CEA expression detected by radiolabeled CEA-specific monoclonal antibody binding. Murine dendritic cells transduced with Ad-CEA were shown to stimulate lymphoblastic transformation of CEA-immune T cells *in vitro*.

Table 5. Dendritic Cell Transduction

	Luciferase reporter gene			
	Recombinant Adenovirus	DNA/Liposome	RNA/Liposome	Adenovirus Conjugate
Human breast cancer	1,000,000	51,000	11,000	50,000
Human T cells	190	32	30	30
Human B cells	180	32	30	31
Human monocytes	990	29	30	29
Human dendritic cells	7,900	31	31	30
Mouse spleen	2,900	41	29	30

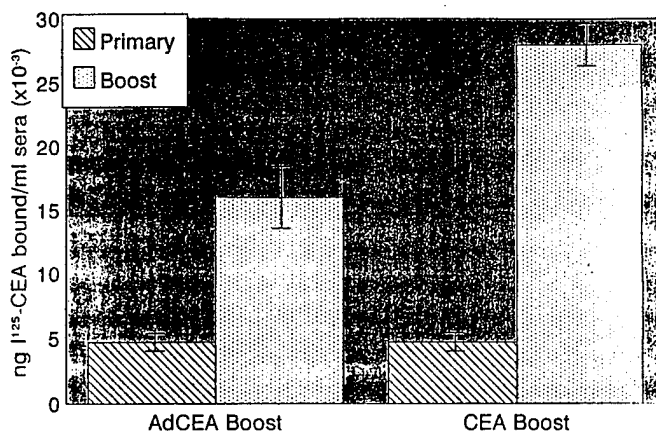
We have begun analysis of CEA gene transduction vaccine efforts by examining the effects of i.v. injection of the Ad-CEA for *in vivo* transduction of antigen presenting cells. As seen in Figure IV, mice given a single dose of i.v. Ad-CEA produced very high antibody responses (4,000-7,000 ng/ml) with anamnestic response to Ad-CEA (10,000-20,000 ng/ml) and even higher if given a booster of CEA protein (35,000-50,000 ng/ml). The same profound antibody response occurred if the vector (Ad-CEA) was given i.p. (Figure V). However, immunoprotection against tumor challenge with syngeneic CEA expressing tumor cells did not occur (Figure VI).

Figure IV. Anti-CEA Ab Response Among Mice Immunized with AdCEA Via I.V. Route



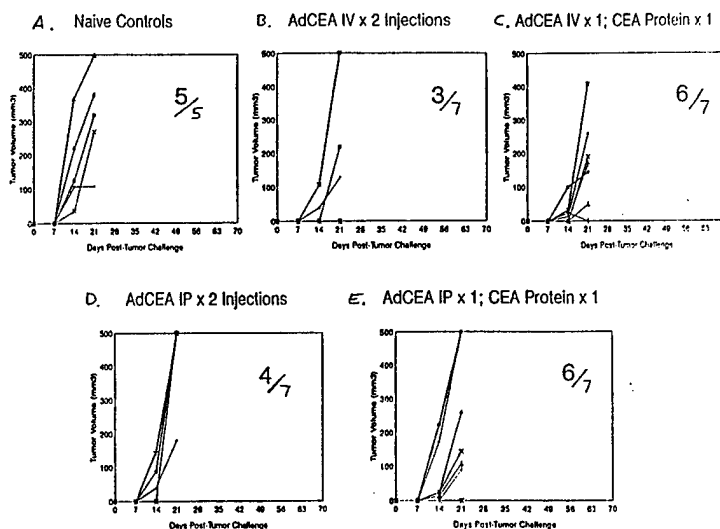
Two groups of seven C57BL/6 mice received 10⁹ pfu of replication incompetent adenovirus encoding CEA (AdCEA) by i.v. injection on day 1. On day 29, one group was boosted with 10⁹ pfu of AdCEA i.v. whereas the other group was boosted with 25 µg of CEA protein by i.m. injection. Mice were bled on days 29 and 43 to assess primary and boosted anti-CEA antibody responses, respectively. Values are the mean ± S.E.M. for seven mice.

Figure V. Anti-CEA Ab Response Among Mice Immunized with AdCEA Via I.P. Route



Two groups of seven C57BL/6 mice received 10⁹ pfu of AdCEA by i.p. injection on day 1. On day 29, one group was boosted with 10⁹ pfu of AdCEA i.p. whereas the other group was boosted with 25 µg of CEA protein by i.m. injection. Mice were bled on days 29 and 43 to assess primary and boosted anti-CEA antibody responses, respectively. Values are the mean ± S.E.M. for seven mice.

Figure VI. Tumor Challenge Outcome Among Mice Immunized With Recombinant Adenovirus Encoding CEA With or Without CEA Protein Boosting



Groups of seven C57BL/6 mice were immunized as follows: 1) 10^9 pfu of AdCEA i.v. on days 1 and 29 (panel B); 2) 10^9 pfu of AdCEA i.v. on day 1 and 25 μ g of CEA protein i.m. on day 29 (panel C); 3) 10^9 pfu of AdCEA i.p. days 1 and 29 (panel D); or 4) 10^9 pfu of AdCEA i.p. day 1 and 25 μ g of CEA protein i.m. day 29 (panel E). These mice as well as five naive controls (panel A) were challenged with 2×10^5 MC38-CEA cells on day 43. Serial tumor measurements and the total number of tumor bearing mice in each group are shown.

We have just begun our analysis of Ad-CEA transfected dendritic cell immunizations. A pilot study has shown six of six mice given i.p. transfected dendritic cells have antibody response to CEA and further studies are ongoing.

Conclusions:

These studies have demonstrated the feasibility of studying freshly isolated human blood or murine splenic dendritic cells. Overnight protein loading generated dendritic cell vaccines which were quite effective at induction of humoral and cellular immunity in a model system (BSA). However, the strategy produced weak immune responses using a tumor antigen (CEA) presumably due to requirements for high concentrations of the loading immunogen. We have developed a means to induce CEA expression in dendritic cells using a gene transfer vector (replication defective adenovirus [Ad-CEA]). The Ad-CEA alone produced intense antibody response to CEA following i.v. and i.p. injection but no tumor protection. Studies are ongoing with Ad-CEA transduced dendritic cells. We plan to expand our observations with both Ad-CEA and Ad-CEA transduced dendritic cells in regards to characterization of the immune response (TH-1 or TH-2 response), studies of antitumor effects in tumor challenge and microscopic metastatic disease setting and to examine strategies to enhance antitumor effects (dual expression vectors for antigen and B7.1, IL-2, etc.). We have also developed an Ad-Erb-2 vector to expand these studies to a second breast cancer relevant system.

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